The EMBO Journal vol.5 no.8 pp.1951-1957, 1986

Functional analysis of the 5′ flanking sequence of a vaccinia virus late gene

Christine Bertholet, Patrick Stocco, Erwin Van Meir and Riccardo Wittek

Institut de Biologie animale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland

Communicated by B.Hirt

A series of mutations, including 5′ and 3′ deletions, as well as insertions were introduced into the 5′ flanking nucleotide sequence of a vaccinia virus late gene. This DNA has been shown previously to contain all the necessary elements for correct regulation of the gene most probably transcribed by the viral RNA polymerase. To facilitate the assays, the mutated DNA was fused to the chloramphenicol acetyltransferase gene and inserted into the genome of live vaccinia virus. The effects of the mutations on expression of the chimeric gene were studied by both enzyme assays and nuclease S1 anlaysis. The results showed that 5′ deletions up to about 15 bp from the putative initiation site of transcription still yielded high levels of gene expression. All mutations, however, that deleted the authentic late mRNA start site, abolished promoter activity.

Key words: gene expression/homologous *in vivo* recombination

Introduction

Vaccinia virus is a member of the poxvirus family and contains a large double-stranded DNA genome of about 190 kb. The genome of most animal DNA viruses migrates into the nucleus of the infected cell where the genes are transcribed by host enzymes. In contrast, vaccinia virus replicates in the cytoplasm and presumably utilizes exclusively virus-encoded enzymes for the expression of its genes (Moss, 1985). Two temporal classes of vaccinia virus genes have been identified: early and late genes, defined as being transcribed before or after the onset of viral DNA replication. Little is known about the molecular mechanism of this gene regulation. Recent studies, however, have indicated that the regulatory elements are contained within the 5′ flanking sequences of the genes (Weir and Moss, 1984; Bertholet *et al.*, 1985; Cochran *et al.*, 1985).

We have recently mapped and sequenced a vaccinia virus late gene encoding a major structural polypeptide and have shown that 100 bp of its 5′ flanking sequence including the late mRNA start site, contains all the necessary information for late gene regulation. This DNA has now been analyzed in more detail and in this communication we show that only about 15 bp of it are sufficient for correct regulation of transcription. All mutations at the RNA start site, however, abolish transcriptional activity.

Results

Construction of plasmids

To identify the regulatory elements contained in the 5′ flanking sequence of a vaccinia virus late gene encoding a structural polypeptide of mol. wt 11 000 (11 kd), we have introduced a series of mutations into this DNA and tested their effects on transcription. As the starting material to construct these mutants we used a recombinant plasmid into which the entire vaccinia virus thymidine kinase (TK) gene (Hruby and Ball, 1982; Bajszar *et al.*, 1983) as well as 5′ and 3′ flanking sequences had been inserted. Into the unique *Eco*RI site located close to the center of the TK gene, a fragment of 100 bp from the 5′ flanking sequence of the 11-kd gene including the mRNA start site had been cloned. Subsequently *Eco*RI and *Bam*HI sites were eliminated. The resulting plasmid was designated pHGS-1 (Figure 1, top).

To create 5′ deletions in the flanking sequence of the late gene, the plasmid was linearized with *Cla*I and then digested with *Bal*31 nuclease. After the addition of *Bam*HI linkers, the plasmid was recircularized and used to transform *Escherichia coli*. Plasmids with the desired deletions were identified after cleavage with *Bam*HI and *Eco*RI. The sequences removed upstream from the *Cla*I site were then replaced by the corresponding intact region isolated from a second plasmid (top right). The precise extent of the deletions was determined by sequencing. These plasmids were then either used to construct the linker scanning mutants (see below) or were used directly to generate vaccinia virus recombinants after insertion of the CAT coding sequences downstream of the remaining 11-kd flanking sequences.

A series of 3′ deletions was generated by first cleaving plasmid pHGS-1 with *Eco*RI and then incubating the DNA with T4 DNA polymerase in the presence of TTP but in the absence of other dNTPs. *Bam*HI linkers were added and the plasmids were recircularized. Clones with the appropriate deletions were selected on the basis of restriction enzyme digestion. The sequences deleted downstream from the *Eco*RI site were replaced with a corresponding region of non-deleted DNA (top left). These plasmids were used to construct the linker scanning mutants and to analyze the effect of deletions around the 11-kd mRNA start site.

Linker scanning mutants (McKnight and Kingsbury, 1982) were constructed by ligating the appropriate *Hin*dIII–*Bam*HI fragments from the 5′ deletion and 3′ deletion mutants to each other. This resulted in the reconstruction of the 11-kd flanking sequences but with a linker inserted at various positions along the DNA (bottom).

Finally, the bacterial CAT gene was inserted downstream of the 5′ deletion and linker scanning mutants of the 11-kd flanking sequences. The gene was isolated and cloned into the *Eco*RI site. These manipulations put the ATG of the CAT gene in frame with the ATG of the 11-kd gene. Thus CAT was expressed in vaccinia virus recombinants (see below) as a fusion protein containing the first four amino acids of the 11-kd polypeptide.

The relevant portion of each plasmid was sequenced and is shown at the top of Figures 2, 3 and 5.

Construction of vaccinia virus recombinants

The possibility to insert foreign DNA into the vaccinia virus genome by homologous *in vivo* recombination provides a powerful means of studying regulatory DNA sequences in an *in vivo* situation. We have therefore used the plasmids described above to

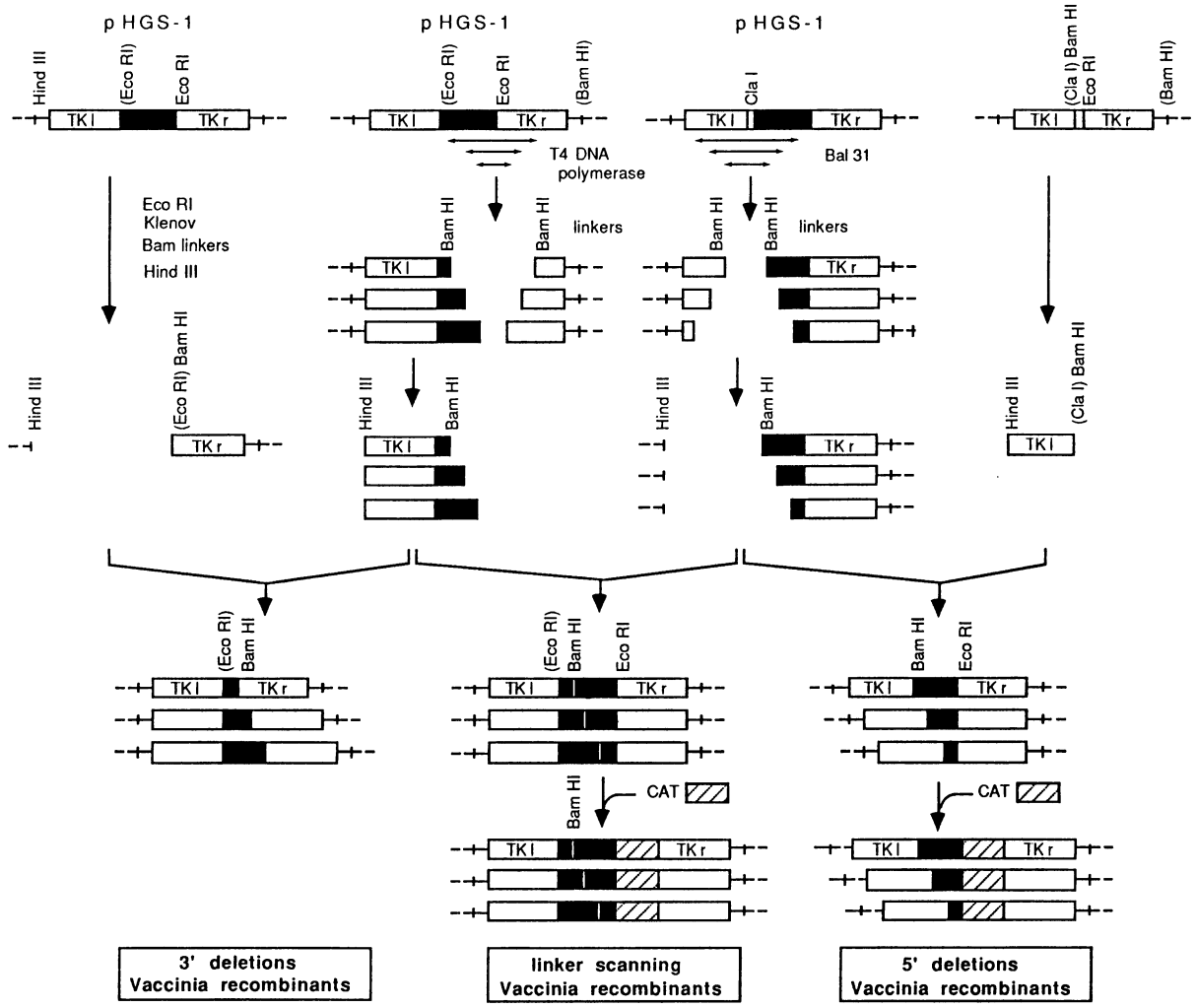

Fig. 1. Strategy for introducing various mutations in the 5′ flanking sequence of the 11-kd gene. The 11-kd flanking sequences inserted into the body of the TK gene are represented by the black box, and the TK gene (l, left part; r, right part) by the open boxes. Restriction sites that were eliminated during plasmid construction are shown in brackets. Note that the pHGS-1 plasmids at the top of each scheme are identical, but that only restriction sites relevant to the particular protocol are shown.

generate such recombinants. Cell cultures were infected with vaccinia virus and then transfected with the appropriate recombinant plasmid DNA. After 48 h the desired recombinants were isolated on the basis of their TK^- phenotype. After two cycles of plaque-purification the DNA from recombinant virus was isolated and characterized by restriction enzyme digestion and dot blot analysis (not shown).

Expression of the CAT gene

To study the effect of various 5′ deletions in the 11-kd flanking sequences on the expression of the CAT gene, cell cultures were infected with recombinant vaccinia virus. At 9 h after infection, cell extracts were prepared and assayed for enzyme activity (Figure 2). As expected, no activity was detected in non-infected cells nor in cells infected with wild-type vaccinia virus. In contrast, recombinants r100, r34, r30, r24, r21 yielded large amounts of enzyme activity although the latter two expressed only about one-third as much as the others. This became obvious only after diluting the cell extracts and counting the radioactivity in the spots in appropriate chromatographs. Deletion of even more sequences (r5 and r1) completely abolished CAT gene expression.

We have also analyzed the effect of insertions at various positions along the 11-kd flanking sequence ('linker-scanning') on expression of the CAT gene (Figure 3). Three of the four constructs tested showed considerable CAT activity. The only cells showing little activity were infected with the mutant r90-1 in which the insertion had replaced the sequences around the 11-kd mRNA start site. This is somewhat in contrast to the result obtained with the 5′ deletion mutants where a significant drop in CAT activity had already occurred before the sequences at the mRNA start site were deleted. It is possible that a high A + T content of the 11-kd flanking sequences is important to obtain high levels of gene expression. Clearly, insertion of a linker alters the overall base composition less than extensive deletions and replacement by TK coding sequences.

Nuclease S1 analysis

The effect of various mutations in the 11-kd flanking sequences was also studied at the RNA level by S1 analysis. Cell cultures were infected with recombinant virus and RNA was isolated early and late in infection. The RNA was then hybridized to one of three 5′ end-labelled DNA probes (Figure 4, top). Probe 1 was a *Hin*dIII−*Cla*I fragment, labelled at the *Cla*I site, that detected RNA made from the TK early promoter. As a further control, probe 3 was used to detect RNA from the 11-kd gene at its authentic position. Transcription from the chimeric 11-kd−CAT

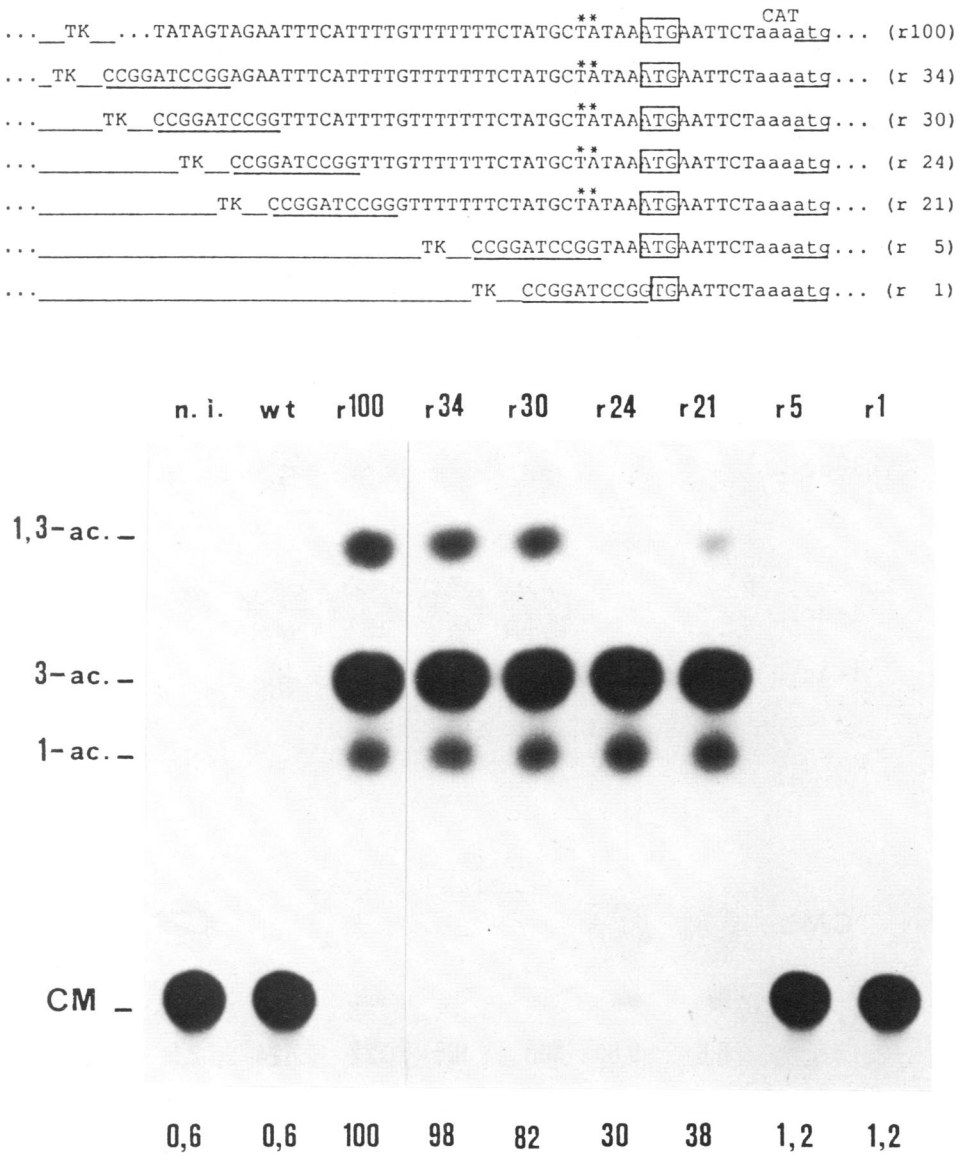

Fig. 2. Assay of CAT activity. CV-1 cells were infected with recombinant vaccinia virus carrying the CAT gene fused to 5′-deleted 11-kd flanking sequences as indicated at the top. The *Bam*HI linker is underlined as is the ATG of the CAT gene (lower case letters). The initiation site of transcription of the 11 kd gene is indicated by the asterisks and the translation initiation codon is shown in the box. Designation of recombinants (r) is based on the number of nucleotides remaining upstream of the G of the ATG codon. Cell extracts were prepared at 9 h after infection and equal amounts of protein were incubated with ^{14}C-labelled chloramphenicol. The reaction products were separated by t.l.c. and the positions of chloramphenicol (CM) and its acetylated forms (1-ac., 3-ac., 1,3-ac.) were detected by autoradiography. The numbers on the bottom line represent the percentage of conversion of CM to its acetylated forms. These were calculated by counting the radioactivity in the spots from an experiment in which the extracts had been diluted to yield less than one-half of the CM in an acetylated form (linear enzyme range). Percentages have been calculated with respect to r100, which was taken as 100%, and they represent the mean values of three independent experiments. n.i., non-infected cells; wt, cells infected with wild-type vaccinia virus.

gene inserted in the TK gene was analyzed with probes 2 labelled at the *Eco*RI site within the CAT gene.

With early RNA hybridized to probe 1, an intense band of 250 nucleotides was obtained indicating transcription from the TK early promoter. As expected, no band was observed with early RNA with probe 3 designed to detect transcription from the 11-kd late gene at its normal position. Unexpectedly, a faint band of 220 nucleotides was observed in all recombinants with probe 2, which detected transcription from the insertions of the late gene flanking sequences. The size of the protected fragment clearly indicated that initiation of transcription does not occur at the RNA start site of the 11-kd gene, but downstream, within the sequence TAAA immediately preceding the ATG of the CAT gene (for the sequence at that position see also Figures 2 and 3).

With late RNA hybridized to probe 1, the band resulting from transcription of the TK gene is no longer observed presumably because early RNAs are present in low amounts late in infection. Instead, a protected fragment of 300 nucleotides is now observed with probe 3, indicating transcription from the 11-kd late gene. The major protected DNA fragment obtained with probe 2 has a size of 220 nucleotides and results from transcription initiation at the CAT gene. This band has already been observed in trace amounts with early RNA. However, late RNA hybridized to probe 2 also protected another fragment of 235 nucleotides indicating transcription from the translocated 11-kd flanking sequences. This band is absent in the 5′ deletion mutants 1 and 5 and in the insertion mutant 90-1 all of which yielded very little CAT activity in the enzyme assay. Apparently, all mutations that

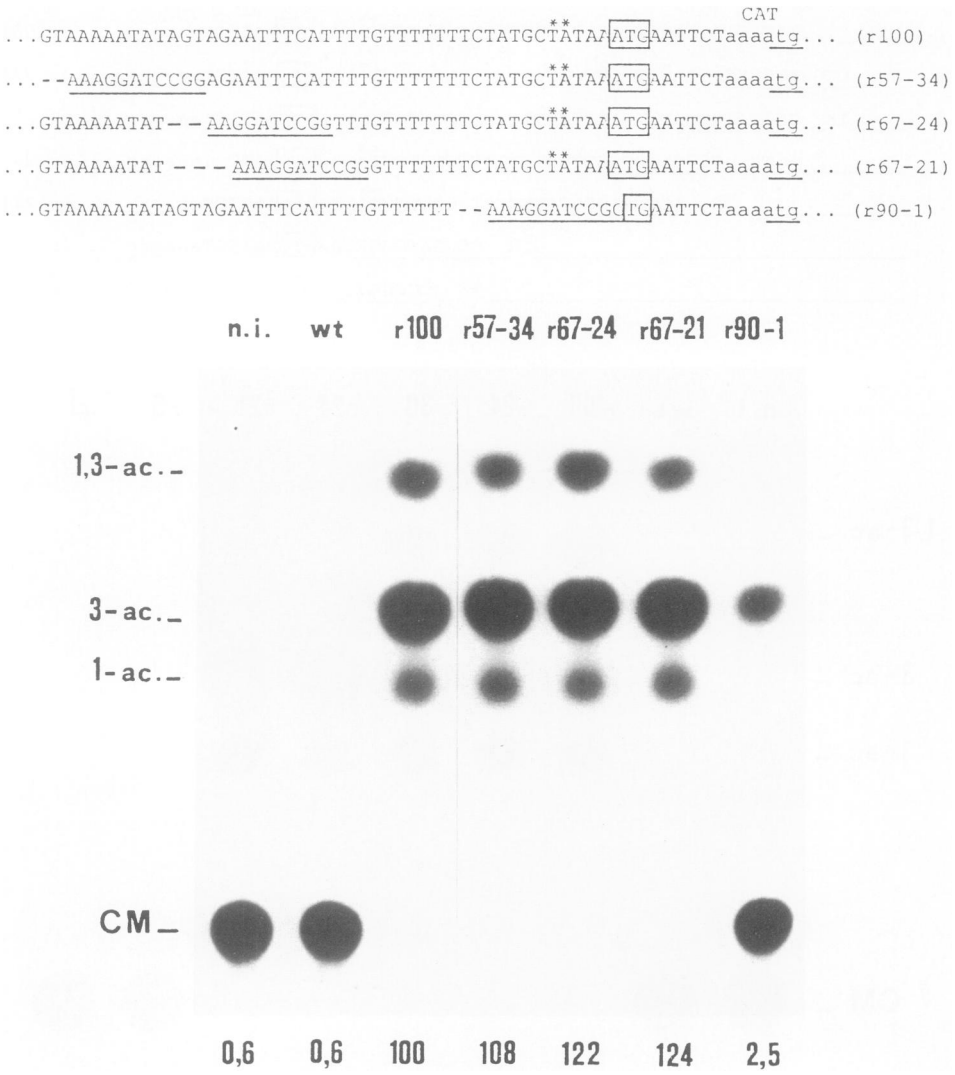

Fig. 3. Assay of CAT activity. CV-1 cells were infected with recombinant vaccinia virus carrying the CAT gene fused to the 11-kd flanking sequences in which a linker (underlined) had been inserted at various positions. The positions of nucleotides that were lost during plasmid construction are indicated by a broken line. Designation of recombinants is based on the number of nucleotides upstream from the linker (first number) and the number of nucleotides between the linker and the G of the ATG codon (second number). For experimental details and abbreviations see legend to Figure 2.

alter the sequence at the initiation site of transcription of the 11-kd gene insertions severely inhibit the synthesis of functional mRNA.

To confirm this result and to rule out the possibility that insertion of the CAT gene interferes with transcription initiation, two further vaccinia virus recombinants were constructed using the 3′ deletion plasmids. Both contain insertions of about 100 bp of 11-kd flanking sequences in the TK gene; however, neither contains the CAT gene. Recombinant A (rA; Figure 5, top) is an insertion mutant which contains the sequence AAAGGATCC inserted between the T and G of the 11-kd ATG translation initiation codon. This insertion resulted from the particular *Bam*HI linkers used to construct the plasmid. Recombinant B (rB) contains the same insertion but is characterized by a deletion of 13 nucleotides comprising the initiation site of transcription. RNA was isolated from cells infected with the two recombinants, both early and late in infection, and analyzed by S1 mapping using 5′ end-labelled probes derived from the corresponding plasmids. Early in infection, a strong signal was obtained from both recombinants with probe 1 detecting transcription from the TK early promoter, but not with probe 2, which detected RNA made from the translocated 11-kd sequences. Late in infection, an intense band was obtained with probe 2 from RNA of recombinant rA. Significantly, however, this band was absent in recombinant rB in which the DNA sequences of the initiation site of transcription had been deleted.

Discussion

The present study was undertaken to identify the regulatory elements previously shown to reside within a maximum of 100 bp of 5′ flanking sequence of a vaccinia virus late gene including the mRNA start site (Bertholet *et al.*, 1985).

A series of mutations were therefore introduced into this fragment, which had been inserted into the body of viral TK early gene. The mutated fragments were fused to the CAT coding sequences and the chimeric gene was then introduced into the genome of live vaccinia virus recombinants by homologous *in vivo* recombination mediated by the TK flanking sequences.

Analysis of CAT expression driven by various 5′ deletion mutants showed that only very short stretches of 5′ flanking sequences are required to obtain high levels of gene expression.

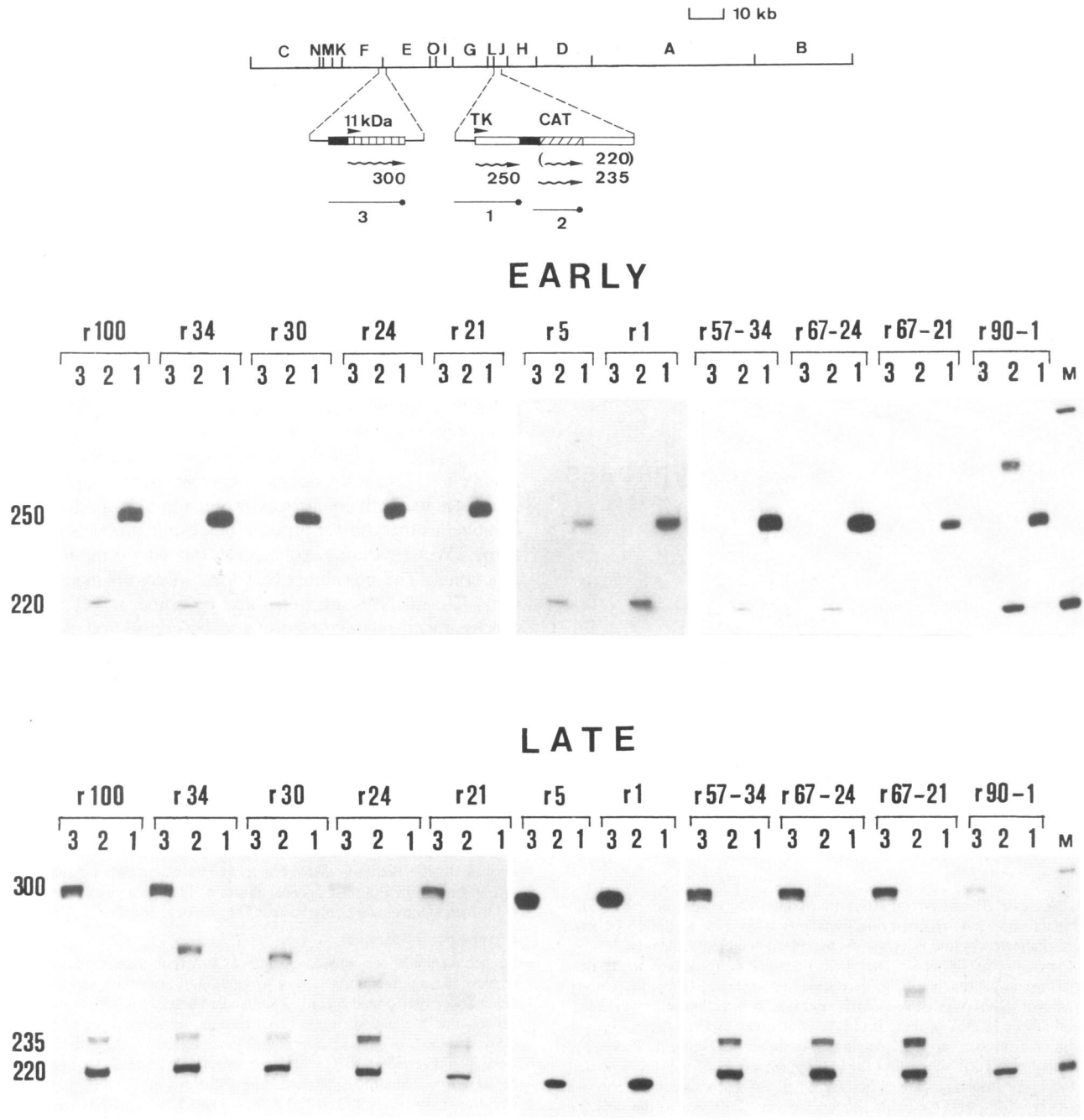

Fig. 4. Nuclease S1 analysis of RNA transcripts. The upper part of the figure shows a *Hin*dIII map of the vaccinia virus DNA and the positions of the 11-kd and TK genes. The coding sequences are indicated by empty or hatched boxes, and the 5′ flanking sequences of the 11-kd gene by the black box. The 5′ end-labelled (solid circles) probes 1 and 3 have been described previously (Bertholet *et al.*, 1985). Probe 2 was labelled at the *Eco*RI site in the CAT gene. The sizes of the nuclease S1-protected fragments (wavy arrows) are also indicated. The unexpected transcript from the CAT gene is shown in brackets. Cells were infected with recombinant vaccinia virus and RNA was isolated at 4 h (early RNA) and at 7 h (late RNA) after infection and hybridized to the three probes. After nuclease S1 digestion, resistant material was resolved in a 6% sequencing gel. The band of decreasing size results from hybridization of read-through transcripts to the homologous part of the probe.

Readily detectable amounts of CAT activity were even expressed by recombinants r24 and r21, which contain only 22 and 19 nucleotides, respectively, upstream from the 11-kd ATG. A significant drop occurred between recombinants r30 and r24. Although others have shown that short stretches of 5′ flanking sequences are sufficient for gene expression in vaccinia virus (Cochran *et al.*, 1985) the novel aspect of the results presented here is the sequence specificity at the RNA start site.

Comparisons of this region between different late genes already characterized reveals two interesting features. First, the 5′ ends of the three late mRNAs map within a few nucleotides of the ATG translation initiation codon (Weir and Moss, 1984; Bertholet *et al.*, 1985; Rosel and Moss, 1985). Second, the sequence TAA immediately precedes the ATG codon in all late genes sequenced (Weir and Moss, 1984; Bertholet *et al.*, 1985; Plucienniczak *et al.*, 1985), with one exception (Rosel and Moss, 1985). Thus, the sequences in the immediate vicinity of the 5′ ends of the RNAs are highly conserved in late genes suggesting some important

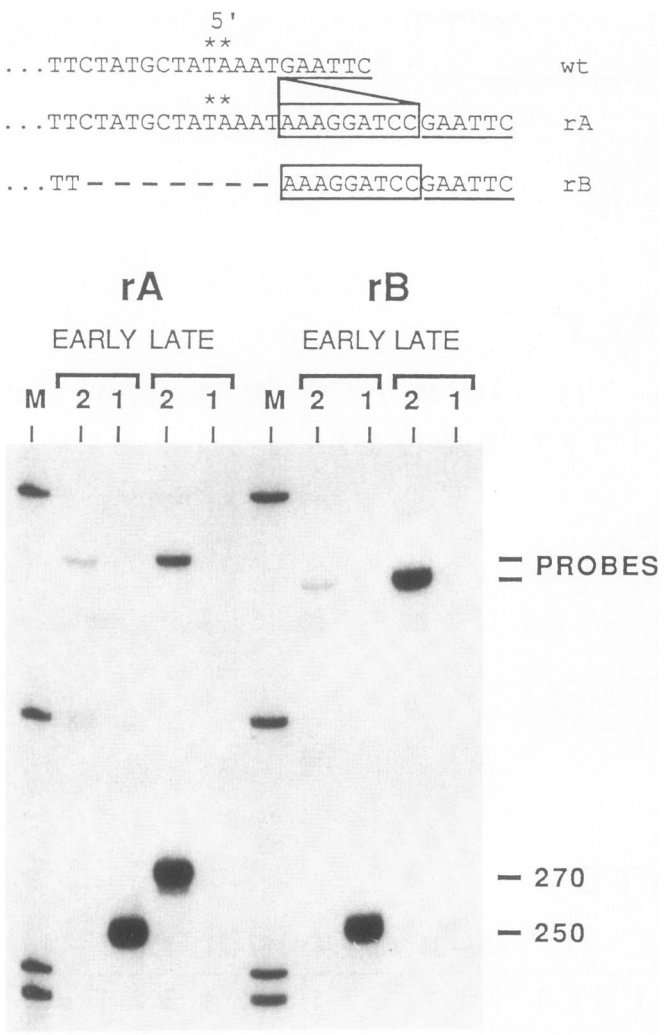

Fig. 5. Nuclease S1 analysis of RNA transcripts. Cells were infected with recombinant virus (rA, rB) carrying mutations at or close to the 11-kd RNA start site (indicated by the asterisk) on the 11-kd flanking sequences translocated into the TK gene. The nucleotides that were deleted in rB are indicated by the broken line. The corresponding sequence in wild-type (wt) is also shown. RNA was isolated early and late in infection and hybridized to 5'-end-labelled DNA probes. Probe 1, which was used to detect transcription from the early TK promoter, is shown in Figure 4. Probe 2 was labelled at a *Taq*I site within the coding sequences of the TK gene and spans the 11-kd insertion. After nuclease S1 digestion, resistant material was analyzed on a 6% polyacrylamide sequencing gel. The sizes of the nuclease S1-protected DNA fragments (in nucleotides) are indicated at the right. M, *Hpa*II fragments of pBR322 used as size markers.

function. This was tested by analyzing several recombinants containing insertions at various positions along the 11-kd flanking sequences. Significantly, recombinant r90-1, in which a linker was inserted at the RNA start site, produced very little CAT activity whereas insertions at other positions had no negative effect. This result was confirmed by S1 analysis of two recombinants (rA and rB, Figure 5) carrying mutations at or close to the 11-kd mRNA start site. Insertion of a 9 bp sequence between the T and G residue of the ATG translation initiation codon has no effect on transcription. However, deletion of a 13-bp sequence also containing the mRNA start site, completely abolished transcription.

This contrasts sharply with the situation observed for cellular and viral genes transcribed by RNA polymerase II. In this case the transcription initiation site is determined by the position of the TATA box located at about 25–30 bp upstream (Breathnach and Chambon, 1981). Unlike with the vaccinia virus late genes, the actual sequence at the RNA start site itself does not appear to be of crucial importance.

Nuclease S1 mapping was also used to confirm the results obtained by CAT assays. As expected in all recombinants that were positive in the enzyme assay, a transcript was detected mapping at the 11-kd mRNA start site. To our surprise, a second RNA, present in even large amounts as judged from the intensity of the bands, was observed in all recombinants, including those negative in the enzyme assay. The size of this RNA indicated that the 5' end maps within the sequence TAAA immediately preceding the ATG of the CAT gene (see Figures 2 and 3, top). Thus it appears that the sequence TAAAATG, which differs from the consensus sequence found in most late genes by only one additional A residue, seemingly created a regulatory element recognized by the vaccinia virus transcriptional machinery. Faint bands observed with early RNA at the corresponding positions of the gel indicate that this element also represents a signal early in infection. This RNA does not represent functional CAT mRNA since it is made in equal amounts also in cells infected with recombinants that did not produce detectable amounts of CAT enzyme. We have no explanation for why this RNA is not functional. The possibility that the 5' ends of mature vaccinia virus late mRNAs are generated by some as yet unidentified processing mechanism, must also be considered. We are currently investigating this intriguing possibility.

Materials and methods

Cells and viruses

Human TK$^-$ 143 B cells (Rhim *et al.*, 1975) were obtained from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ; repository no. GM 5887) and grown as described (Bertholet *et al.*, 1985). CV-1 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

The WR strain of vaccinia virus was obtained from B.Moss, National Institutes of Health, Bethesda, MD. The temperature-sensitive mutant ts 7 (Drillien and Spehner, 1983) of the Copenhagen strain of vaccinia virus was obtained from R.Drillien, Université Louis Pasteur, Strasbourg, France.

Construction of plasmids

Routine manipulations, such as cloning of DNA restriction fragments, purification of plasmids and the elimination or generation of restriction sites using synthetic linkers, were performed according to standard procedures (Maniatis *et al.*, 1982). To generate 5' deletions in the 11-kd flanking sequences, plasmid pHGS-1 was cleaved at the *Cla*I site (see also Figure 1) within the TK gene and 30 μg of DNA were then digested at 16°C with three units of *Bal*31 nuclease in a total volume of 300 μl of standard reaction mixture (Maniatis *et al.*, 1982). Aliquots were withdrawn after 0, 1, 2, 2.5, 3, 3.5, 4, 4.5 and 5 min and the extent of digestion was determined by polyacrylamide gel electrophoresis after cleaving 100 ng of DNA with *Eco*RI and labelling the ends. DNA digested for 2, 2.5, 3 min and 3.5, 4, 4.5 min, respectively, was pooled and *Bam*HI linkers (CCGGATCCGG) were ligated to the ends. Further steps in plasmid construction are depicted in Figure 1 and described in Results.

To obtain 3' deletions in the 11-kd flanking sequences, plasmid pHGS-1 was cleaved with *Eco*RI and 3 μg were incubated for 5 min at 37°C with one or three units of T4 DNA polymerase in the presence of 100 μM dTTP. Each of the two samples was then split into three aliquots which were treated with 4, 8 and 16 units of S1 nuclease. *Bam*HI linkers (AAAGGATCCTTT) were then ligated to the ends, the DNA was cleaved with *Bam*HI, recircularized by ligation and used to transform *E. coli*. Clones wtih the desired deletions were identified after cleavage with *Cla*I and *Bam*HI and amplified. These plasmids were used to construct the linker scanning mutants as shown in Figure 1, or used directly to generate recombinant vaccinia virus after replacement of the deleted TK sequences as also outlined in Figure 1.

The linker scanning mutants were constructed as shown in Figure 1 and described in the text (Results). Since different *Bam*HI linkers were used to construct the 5' and 3' deletions, the 11-bp linker insertion (AAAGGATCCGG) in the final constructs is asymmetric.

The CAT gene, which was inserted downstream from the 5' deletion and linker scanning mutants, was isolated from a plasmid described by Stueber and Bujard

(1982) as an *Alu*I partial digestion product of 823 bp from an *Alu*I cleavage site at 5 bp upstream of the ATG translation codon to an *Alu*I site at 158 bp downstream of the TAA translation stop codon. The gene was ligated into the filled-in *Eco*RI site downstream of the 11-kd ATG.

Construction of recombinant vaccinia virus

The mutated 11-kd flanking sequences either alone (3′ deletions) or fused to the CAT coding sequences (5′ deletions and insertion mutants) were inserted into the DNA of live vaccinia virus recombinants by homologous *in vivo* recombination as described (Bertholet *et al.*, 1985), except that the CV-1 cells were used for transfection.

CAT assay

CV-1 monolayer cultures were infected with 10 plaque-forming units per cell of recombinant vaccinia virus. At 9 h after infection, cell extracts were prepared and assayed for CAT activity as described (Gorman *et al.*, 1982).

Isolation of RNA

Early and late RNA was isolated from infected CV-1 monolayers as described (Wittek *et al.*, 1984).

Nuclease S1 analysis

RNA was mapped by S1 nuclease analysis (Berk and Sharp, 1977) using 5′ end-labelled DNA probes (Weaver and Weissman, 1979). Hybridizations were carried out at 42°C and single-stranded nucleic acids were digested at 25°C with 20 units/ml of S1 nuclease (P-L Biochemicals).

The probes used to detect RNA from the TK early and from the 11-kd late gene at its authentic position have been described (Bertholet *et al.*, 1985). The probes for detection of RNA made from the chimeric CAT gene were prepared as follows. Plasmid pHGS-1 containing the CAT gene was partially cleaved with *Eco*RI, end-labelled and then cleaved with *Hin*dIII. The DNA fragment from the *Hin*dIII site to the *Eco*RI site 210 bp downstream of the ATG of the CAT gene was isolated and used as the hybridization probe for all recombinants, except for recombinants r1, r5 and r90-1. In these recombinants the mutation had eliminated the sequences at the authentic initiation site of transcription and S1 probes were therefore made for each recombinant from the corresponding plasmid DNA in the same manner as described above.

The probes for the recombinants carrying mutations at the 3′ end of the 11-kd flanking sequences (recombinants rA and rB) were made for each recombinant from the corresponding plasmid DNA. The probe was constructed by first cloning a *Hin*dIII partial *Taq*I digestion product from the *Hin*dIII site upstream of the TK gene (see also Figure 1) to a *Taq*I site closest to the end of the coding sequences of the TK gene (Hruby *et al.*, 1983; Weir and Moss, 1983). Cloning of these fragments into the *Hin*dIII–*Cla*I site of pBR322 created a convenient unique *Cla*I site. The plasmids were linearized with *Cla*I, end-labelled, cleaved with *Hin*dIII and the fragment containing the TK gene and inserted 11-kd flanking sequences was isolated and used as the probe.

Acknowledgements

We thank Claude Mancini for technical assistance, Henk Stunnenberg for plasmids and valuable discussions, Bob Hipskind, Anne Seiler-Tuyns and Walter Wahli for critically reading the manuscript, Pierre Hirt for help in preparing the illustrations and Hannelore Pagel for secretarial help. This work was supported by the Swiss National Science Foundation.

References

Bajszar,G., Wittek,R., Weir,J.P. and Moss,B. (1983) *J. Virol.*, **45**, 62–72.
Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–723.
Bertholet,C., Drillien,R. and Wittek,R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2096–2100.
Breathnach,R. and Chambon,P. (1981) *Annu. Rev. Biochem.*, **50**, 349–383.
Cochran,M.A., Puckett,C. and Moss,B. (1985) *J. Virol.*, **54**, 30–37.
Drillien,R. and Spehner,D. (1983) *Virology*, **131**, 385–389.
Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
Hruby,D.E. and Ball,L.A. (1982) *J. Virol.*, **43**, 403–409.
Hruby,D.E., Maki,R.A., Miller,D.B. and Ball,L.A. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3411–3415.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY.
McKnight,S.L. and Kingsbury,R. (1982) *Science*, **217**, 316–324.
Moss,B. (1985) In Fields,B.N., Chanock,R.M. and Roizman,B. (eds), *Virology*. Raven Press, New York, pp. 685–703.
Pluciennicczak,A., Schroeder,E., Zettelmeissl,G. and Streek,R.E. (1985) *Nucleic Acids Res.*, **13**, 985–998.
Rhim,J.S., Cho,H.Y. and Huebner,R.J. (1975) *Int. J. Cancer*, **15**, 23–29.
Rosel,J. and Moss,B. (1985) *J. Virol.*, **56**, 830–838.
Stueber,D. and Bujard,H. (1982) *EMBO J.*, **1**, 1399–1404.
Weaver,R.F. and Weissman,C. (1979) *Nucleic Acids Res.*, **7**, 1175–1193.
Weir,J.P. and Moss,B. (1983) *J. Virol.*, **46**, 530–537.
Weir,J.P. and Moss,B. (1984) *J. Virol.*, **51**, 662–669.
Wittek,R., Hänggi,M. and Hiller,G. (1984) *J. Virol.*, **49**, 371–378.

Received on 9 April 1986

Note added in proof

Hänggi *et al.*(1986) have recently published a similar study but have interpreted their results in the context of a classical eukaryotic promoter, i.e. involving a TATA box and transcription initiation. In contrast we have preferred, in the discussion of our results, to leave the possibility open that vaccinia virus may use a unique mechanism to generate late mRNAs. [Hänggi,M.,Bannwarth,W. and Stunnenberg,H.G. (1986)*EMBO J.*, **5**, 1071–1076.]